Isolation of Antitumor Alkaloids from *Cephalotaxus harringtonia*

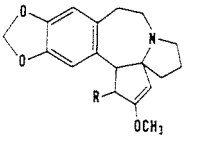
Richard G. Powell,* S. Peter Rogovin, and Cecil R. Smith, Jr.

Northern Regional Research Laboratory, Peoria, Ill. 61604

Purified antitumor alkaloids (harringtonine, isoharringtonine, and homoharringtonine) from *Cephalotaxus harringtonia* have been isolated in the pilot plant. Major processing steps included extraction of the plant material with ethanol, isolation of a crude alkaloid fraction by chloroform extraction, preliminary separation of the crude alkaloids by 10-tube countercurrent distribution, concentration of the active alkaloids by column chromatography, and final separation of the active alkaloids by 200-tube countercurrent distribution. From 455 kg of plant material, about 330 g of crude alkaloid was obtained which yielded 36 g of three purified active cephalotaxine esters.

Several alkaloids isolated from the evergreen tree, *Cephalotaxus harringtonia*, have shown significant activity against L-1210 or P388 leukemia in mice (Mikolajczak, *et al.*, 1972; Powell, *et al.*, 1972). The active alkaloids, which are esters of cephalotaxine (I), include harringtonine (II), isoharringtonine (III), homoharringtonine (IV), and deoxyharringtonine (V). These and a number of other *Cephalotaxus* alkaloids are found throughout the tree but are most concentrated in the seed and are found in lesser amounts in roots, stems, and leaves (Perdue, *et al.*, 1970). Large quantities of seed have been unavailable for processing. Leaves also contain high concentrations of lipids and other ethanol-soluble materials which form tenacious emulsions that are difficult to process.

Alkaloids II and IV have both been cleared for preclinical pharmacological evaluation by the National Cancer Institute on the basis of their initial activity. Alkaloid IV is currently favored over alkaloid II as it shows activity at



I: R = –OH

II: R = $CH_3C(CH_2)_2CCH_2CO_2CH_3$ (OH, CH_3, OH, CO_2–)

III: R = $CH_3CH(CH_2)_2C$–$CHCO_2CH_3$ (OH, CH_3, OH, CO_2–)

IV: R = $CH_3C(CH_2)_3CCH_2CO_2CH_3$ (OH, CH_3, OH, CO_2–)

V: R = $CH_3CH(CH_2)_2C$–$CH_2CO_2CH_3$ (OH, CH_3, CO_2–)

Reprinted from I&EC PRODUCT RESEARCH & DEVELOPMENT, Vol. 13, Page 129, June 1974

somewhat lower dose levels and is available in larger quantity.

This paper describes the pilot-plant process used to obtain gram quantities of three highly purified antitumor alkaloids from *Cephalotaxus* plant material. The process was developed to provide quantities of the alkaloids for preclinical testing and to provide a guide for projected larger scale preparations.

Experimental Section

General Procedures. The major process steps included: (a) chopping and grinding of defoliated trees, including roots, to a particle size suitable for solvent extraction; (b) extraction of the plant material with 95% ethanol (EtOH); (c) concentration of the extract by evaporation followed by dissolution in aqueous acid and then extraction with chloroform ($CHCl_3$) to remove lipids and other interfering materials; (d) basification and extraction of crude alkaloids from the basic solution with $CHCl_3$; (e) preliminary separation of the alkaloids by a 10-tube countercurrent distribution (CCD) between $CHCl_3$ and McIlvaine's buffer; (f) concentration of the active alkaloids by column chromatography; and (g) purification of the active alkaloids (II, III, and IV) by CCD in a 200-tube apparatus. The entire process is outlined in Figure 1. All extractions were done at room temperature (27 ± 2°C), and solvent evaporations were carried out under reduced pressure at 25–40°C.

A commercial nursery in Oregon provided 25 trees, *Cephalotaxus harringtonia* (Forbes) K. Koch var. *harringtonia* cv. Fastigiata. The trees were harvested in January and dried at room temperature until June before grinding. The roots and lower branches were first cut into short pieces which were cut to 6.5-cm chips with an Appleton chipper. Upper branches were cut into 2.5- to 5.0-cm pieces in a Taylor-Stiles cutter. Final grinding of the chipped material and of the green foliage to *ca.* 0.7-cm chips was done with an attrition mill, waveline plate pattern 61-cm double disk, 1800 rpm each disk, driven by two 50-hp motors (Bauer Bros., Springfield, Ohio).

Ground material was steeped with 95% ethanol (EtOH) in covered 210-l. stainless steel tanks; EtOH, metered volumetrically, was added and decanted through a stainless steel pump. The steep liquids were filtered through a horizontal plate filter (Sparkler) before being concentrated in a stainless steel single-effect, natural circulation, rising film evaporator to remove ethanol.

Extractions with $CHCl_3$ were done in a 245-l. stainless steel tank. A stainless steel pump with Teflon packing and flexible copper hose pumped the $CHCl_3$. A dial-scale measured the $CHCl_3$, and the $CHCl_3$ and aqueous layers were mixed with an air-driven stirrer. The initial 10-tube CCD of crude alkaloids was carried out in 19-l. carboys. $CHCl_3$ extracts were concentrated in a single-effect evaporator (Precision Scientific).

Column chromatography of countercurrent fractions was done in a 5 × 70-cm glass column fitted with a Teflon stopcock. Columns were packed with Woelm activity grade III neutral alumina (50 g/g of alkaloid mixture).

All stages of the alkaloid separation were monitored by thin-layer chromatography (tlc) on precoated 20 × 20-cm Silica Gel F-254 plates, 0.25 mm thickness (Brinkmann Instruments, Inc.). Plates were developed with CH_3OH–$CHCl_3$ (15:85 v/v), and spots were visualized with iodine vapor. Analyses of extracts and concentrates at various stages of isolation were made with a general extraction procedure for crude alkaloids reported earlier (Powell, 1972).

Final separation of alkaloid fractions rich in harringtonine, isoharringtonine, and homoharringtonine was done in a 200-tube Craig–Post apparatus (40 ml of both upper and lower phase per tube). Purity of the isolated alkaloids was checked by infrared spectrometry with a Perkin-Elmer Model 137 instrument (1% solutions in $CHCl_3$) and by nuclear magnetic resonance with a Varian HA-100 instrument ($CDCl_3$ solutions).

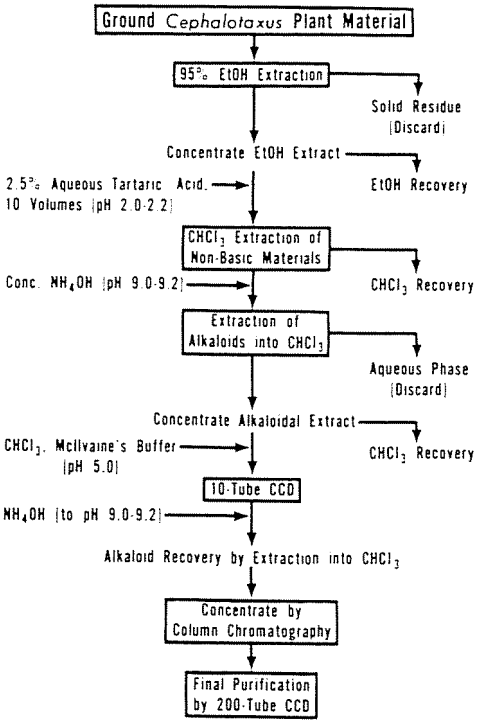


Figure 1. Schematic flow diagram for producing purified antitumor alkaloids from *Cephalotaxus* plant material

Detailed Procedures and Results

Extraction of Plant Materials. Ethanol extraction of ground *Cephalotaxus* plant material was carried out in two batches. The first batch consisted of 240 kg of ground upper and lower branches of the plant minus leaves (tree portion A); by analysis portion A contained 97 g of alkaloids. The plant material was divided equally among 10 containers, and sufficient 95% EtOH (3.8 l./kg of tree) was added to cover it. After the plant material steeped for 48 hr, the EtOH extract was decanted by pump and filtered. This procedure was repeated two additional times but with 1.6 l. of EtOH/kg of tree required for each steep. Residues were analyzed for remaining recoverable alkaloid after each steeping and three steeps removed about 95% of the extractable alkaloids. The EtOH extracts (1210 l.) were combined and evaporated to a volume of 14.1 l.

Tree portion B, 215 kg of lower branches and roots, was extracted similarly. The first steep required 3.8 l. of EtOH/kg of plant material. Three additional steeps using 1.9 l. of EtOH/kg of tree were necessary for nearly complete extraction of the alkaloids. The EtOH extracts (1515 l.) were combined and concentrated to a volume of 13.9 l. By analysis of the concentrates, portion A contained *ca.* 85 g of total recoverable alkaloid and portion B contained *ca.* 250 g.

Isolation of Crude Alkaloid. The concentrated EtOH extract of tree portion A was dissolved in 140 l. of 2.5% aqueous tartaric acid solution and the acidic solution was then gently stirred together with 32 l. of $CHCl_3$. Vigorous stirring was avoided to prevent formation of stable emulsions. The layers were allowed to separate for 1 hr before the lower phase ($CHCl_3$) was removed. Three additional

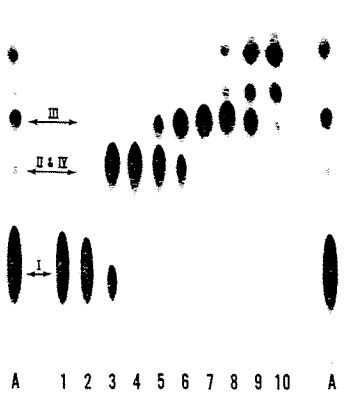


Figure 2. Thin-layer chromatography of *Cephalotaxus* crude alkaloid mixture and of fractions after a 10-tube countercurrent distribution of the mixture: A, crude alkaloid mixture; 1–10, fractions obtained after countercurrent distribution of A. In order of increasing R_f, the spots represent alkaloids I, a mixture of II and IV, III, and other alkaloids

28-l. $CHCl_3$ extractions were made. This process removed lipids and other relatively nonpolar neutral and acidic materials leaving any basic alkaloids in the aqueous phase. The aqueous acidic solution was then made basic to pH 9.1 by addition of 3.45 l. of concentrated NH_4OH. Alkaloidal materials were recovered from the basic solution with five successive 28-l. $CHCl_3$ extractions. Settling periods of 22 hr were necessary between each extraction. Combined $CHCl_3$ extracts were passed through No. 1 Whatman filter paper and evaporated to a volume of 1.5 l. The $CHCl_3$ extract of tree portion A contained 74 g of alkaloids (87% recovery).

The ethanol extract of tree portion B was treated in a fashion similar to portion A, except that the initial extraction involved four 28-l. portions of $CHCl_3$. Combined $CHCl_3$ extracts were concentrated to 4.0 l. and contained *ca.* 253 g of alkaloid mixture (*ca.* 100% recovery).

Preliminary Separation of Alkaloids by 10-Tube CCD. The combined alkaloid concentrates from tree portions A and B, 330 g, was diluted with $CHCl_3$ to 14 l. and half of this amount was placed in the first CCD "tube" (one of 10 carboys) along with 7 l. of McIlvaine's buffer, pH 5 (McIlvaine, 1921). During the entire procedure, the aqueous buffer was the stationary phase and $CHCl_3$, the mobile phase. Buffer and $CHCl_3$ were mixed thoroughly by mechanical agitation after each transfer, the layers were allowed to separate, and the $CHCl_3$ layer was pumped to the next higher tube. After nine transfers the alkaloids were distributed throughout all 10 tubes. The contents of each were then made basic, pH 9.0–9.3, with 364 ml of concentrated NH_4OH per tube; the contents of each were stirred thoroughly; the layers were allowed to separate; and the $CHCl_3$ layers were drawn off. The aqueous layers were washed twice with 2 l. of $CHCl_3$. The entire process was then duplicated with the remaining 7 l. of crude alkaloid concentrate, and appropriate fractions from the two runs were combined.

The combined $CHCl_3$ extracts from the tube 1 gave, upon evaporation to dryness, 128.3 g of alkaloid. Tube 2 yielded 47.7 g; tube 3, 12.6 g; tube 4, 12.2 g; tube 5, 13.4 g; tube 6, 14.4 g; tube 7, 18.3 g; tube 8, 22.0 g; tube 9, 25.8 g; and tube 10, 20.7 g of alkaloid. Thus a total of 315 g of alkaloid was recovered out of the original 330 g of mixed alkaloid subjected to CCD (95% recovery).

Results of the 10-tube CCD are more clearly depicted by the tlc plate in Figure 2. Tubes 1 and 2 contained nearly pure cephalotaxine (176 g). Harringtonine and

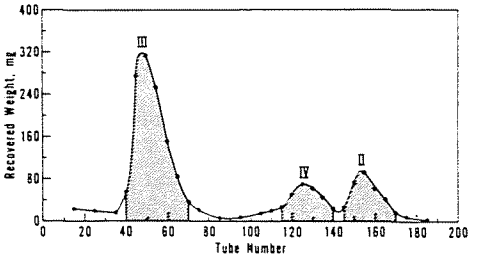


Figure 3. The 200-tube countercurrent distribution of a 9.84 g mixture of alkaloids II–IV. Solvent system, $CHCl_3$–McIlvaine's buffer, pH 5. Shaded areas represent fractions combined to obtain alkaloids of high purity

Table I. Column Chromatography of Fractions from a 10-Tube Countercurrent Distribution (CCD) of *Cephalotaxus* Alkaloids

CCD fraction and weight	Column fractions pooled	Recovered weight, g	Alkaloids present[a]
3 (12.6 g)	1–16	0.18	...
	17–28	2.71	I
	29–32	0.26	...
	33–48	5.15	II, IV
	Column wash	1.22	...
4 (12.2 g)	1–30	0.63	...
	31–48	6.88	II, IV
	Column wash	1.69	...
5 (13.4 g)	1–30	0.46	...
	31–32	0.31	III
	33–44	6.68	II, IV
	45–48	0.12	...
	Column wash	1.49	...
6 (14.4 g)	1–30	0.47	...
	31–34	1.68	III
	35–42	4.35	III[b]
	43–48	0.48	III[b]
	Column wash	2.22	...
7 (18.3 g)	1–26	1.15	...
	27–48	8.43	III[b]
	Column wash	3.26	...
8A (11.0 g)	1–30	1.36	...
	31–50	3.84	III[b]
	Column wash	2.03	...
8B (11.0 g)	1–32	1.44	...
	33–50	3.51	III[b]
	Column wash	1.87	...

[a] Blank spaces represent mixtures of several alkaloids not considered further in this paper. [b] These fractions also contained minor amounts of II and IV.

homoharringtonine, inseparable by tlc, were concentrated in tubes 3 to 6 and there was, in addition, partial resolution of these two alkaloids. Isoharringtonine was found mainly in tubes 6 to 9 with some overlap into tubes 5 and 10. Mixtures of several homoerythrina and other alkaloids were also present in tubes 8 to 10.

Column Chromatography of Countercurrent Fractions. Fractions 3 to 8 from the 10-tube CCD were each dissolved in a minimum of benzene–ethyl ether (4:1 v/v) and chromatographed on neutral alumina to gain further enrichment of the active alkaloids II–IV. The columns were eluted stepwise with 1.5 l. of benzene–ethyl ether (4:1 v/v), 1.5 l. of ether, 1.5 l. of MeOH in ether (1:19 v/v), and 1.5 l. of MeOH in ether (1:3 v/v). Columns were then washed with 1 l. of 10% aqueous acetic acid followed by 0.5 l. of H_2O. Fractions of 125 ml each were collected and after examination by tlc, appropriate fractions were combined. Countercurrent fraction 8 was divided and chromatographed in two separate 11-g portions (8A and 8B). Results of the various column runs are summarized in Table I. Under this set of chromatographic conditions,

Table II. Yields of Various Alkaloids from 445 kg of Dried *Cephalotaxus harringtonia* Plant Material

Alkaloid	Amount, g	Total crude alkaloid, %	Total plant material, %
Total alkaloids	330.0	100.0	0.0720
Harringtonine (II)	5.3	1.6	0.0012
Isoharringtonine (III)	14.4	4.4	0.0032
Homoharringtonine (IV)	16.6	5.0	0.0037

alkaloid I is eluted first, followed by III, and then by a mixture of II and IV.

Separation of Alkaloids II, III, and IV by CCD. Fractions from each column run were pooled on the basis of composition (Table I). Further combinations of alkaloids II–IV were then made to give four fractions as follows: (1) 12.03 g (mixture of II and IV); (2) 10.90 g (III with a small amount of IV); (3) 11.03 g (nearly equal amounts of II, III, and IV); and (4) 7.35 g (mainly III). Each of these fractions was purified further by CCD. Fraction 1, in $CHCl_3$, was divided equally among the first 10 tubes of a 200-tube CCD apparatus. Chloroform was the stationary phase and McIlvaine's buffer, pH 5, was the upper phase (40 ml of each phase per tube). Settling periods of 10 min were used between transfers. In this system, II migrated ahead of IV, and IV moved ahead of III. Fractions 2 and 3 were distributed similarly and after each distribution (190 transfers) a weight plot was obtained. Alkaloids were isolated by making the tube contents basic with NH_4OH and extracting them into $CHCl_3$.

Results of the first three distributions were as follows. (1) The combined contents of tubes 101–134 yielded 6.02 g of IV and tubes 146–179 gave 3.88 g of II. Some overlap was present in tubes 135–145, which yielded 1.15 g of a mixture of II and IV. (2) Tubes 40–70 gave 7.89 g of III and tubes 110–140 afforded 1.48 g of IV. The absence of alkaloid II in this run reflects a partial separation of II and IV achieved by the previous 10-tube CCD. (3) Tubes 46–75 yielded 0.77 g of III and tubes 91–135 gave 7.98 g of IV. A mixture of II and IV, 1.34 g, was recovered from tubes 136–174.

The final distribution was carried out on fraction 4 adding to it the mixtures of II and IV left over from distributions of 1 and 3. Distribution of this final sample, 9.84 g, gave 5.76 g of III (tubes 40–70, 1.15 g of IV (tubes 116–140), and 1.38 g of II (tubes 146–170). A weight plot of this separation is shown in Figure 3 as an example of the separations achieved.

Yields and percentages of total alkaloids and of purified active alkaloids from 455 kg of dried *C. harringtonia* plant material are summarized in Table II. In addition to these, 179 g of relatively pure I was isolated.

Conclusions

On the basis of our results, it is technically feasible to isolate 100-g quantities of IV either by scale-up of the process outlined or by carrying out the isolation batchwise. Several hundred grams of IV are needed for preliminary clinical trials. At present *Cephalotaxus* plant extracts are the only practical source of IV or of any of the active esters of I.

Acknowledgment

We are indebted to Dr. R. E. Perdue, USDA, Beltsville, Md., for procurement of the *Cephalotaxus harringtonia* plant material, to Drug Research and Development, National Cancer Institute, Bethesda, Md., for purchase of solvents needed in large quantities, and to Mrs. M. H. Rawls and Mr. Lloyd Carlson for technical assistance.

Literature Cited

McIlvaine, T. C., *J. Biol. Chem.*, **49**, 183 (1921).
Mikolajczak, K. L., Powell, R. G., Smith, C. R., Jr., *Tetrahedron*, **28**, 1995 (1972).
Perdue, R. E., Jr., Spetzman, L. A., Powell, R. G., *Amer. Hort. Mag.*, 19 (Winter, 1970).
Powell, R. G., *Phytochemistry*, **11**, 1467 (1972).
Powell, R. G., Weisleder, D., Smith, C. R., Jr., *J. Pharm. Sci.*, **61**, 1227 (1972).

Received for review July 27, 1973
Accepted December 10, 1973

The Northern Regional Research Laboratory is part of the Agricultural Research Service, U. S. Department of Agriculture. Mention of firm names or commercial products does not constitute an endorsement by the U. S. Department of Agriculture.